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THE ACTION OF CALCIUM IONS ON POTASSIUM CONTRACTURES OF SINGLE MUSCLE FIBRES

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Calcium ions are considered to be necessary in reactions linking the electrical and mechanical events in muscle fibres (reviewed by Sandow, 1952). But, whereas the effect of calcium ions on excitable membranes (reviewed by Shanes, 1958) and muscle 'models' (reviewed by Hasselbach, 1962) have been fully investigated during the last ten years, less is known about how external calcium influences the process which controls the development of tension in skeletal muscle fibres. This paper aims at giving some information about the properties of this system, which is regarded as the first of several links coupling excitation and contraction. Single fibres were used to achieve a quick change of the external ion concentrations, since a delay would have impeded the analysis of the time course of the transient contractures in twitch fibres.

A preliminary account of this work already has appeared (Lüttgau, 1962).

METHODS

Single fibres were isolated from the semitendinosus or iliofibularis muscles of *Rana temporaria*. After the dissection each fibre was left for about 1 hr in Ringer's solution and tested for excitability before being transferred from the dissection dish to the experimental cell. This transfer took place on a small glass plate, and movement of the fibre through an air–water interface was carefully avoided.

Most of the experiments were carried out with the Perspex cell described by Hodgkin & Horowicz (1959). In early experiments a similar cell with a two-channel tap for changing the solutions was used. One tendon of the fibre was gripped in a Perspex clamp and the other was connected to a mechano-electrical transducer (RCA 5734) by a silver wire of 50 μ diameter. The fibre was stretched to 1·25 times slack length. A sarcomere distance of about 2·8 μ was measured on some fibres under this condition. This was made at the end of the experimental procedure with a microscope fitted with a water-immersion objective. The lengths of the fibres varied from 1·0 to 2·5 cm and the diameter (stretched) from 50 to 130 μ.

The flow rate was adjusted so that all the fluid in the cell was replaced at least every half second. Membrane potentials were measured with intracellular micro-electrodes of the Ling–Gerard type.

Solutions. (Table 1) The fibres were dissected in a phosphate-buffered Ringer's solution (Adrian, 1956). During the experiments solutions containing 4 mM bicarbonate gassed

with 99 % O_2 and 1 % CO_2 were used. In some experiments sodium was replaced by choline to avoid propagated action potentials after the addition of solutions with high potassium. Contractures of twitch fibres were normally induced by solutions in which Na or choline was partly or fully replaced by K (mixtures of solutions *B*, *C*, and *D*). In several experiments, however, the potassium concentration was raised above the isotonic concentration to 190 mM (solution *E*). The contracture solution for slow fibres contained 100 mM-KCl in addition to the normal ionic composition of Ringer's solution (solution *B*). The water used passed through an ion-exchange column and was glass-distilled afterwards. No special precautions were taken in preparing the Ca-free solutions. Experiments were carried out at room temperature (17–24° C).

TABLE 1. Composition of solutions (mg ions/l. solution)

	Na^+	K^+	Choline$^+$	Cl^-	HCO_3^-	HPO_4^{2-}	$H_2PO_4^-$	Ca^{2+}
A	120	2·5	—	121	—	2·15	0·85	1·8
B	119	2	—	117	4	—	—	*
C	2	2	117	117	4	—	—	*
D	2	102	17	117	4	—	—	*
E	2	190	—	188	4	—	—	*
F	119	102	—	217	4	—	—	*

* Ca was added as $CaCl_2$ in solutions *B* to *F*; its concentration varied from 0 to 15 mM (see text).

RESULTS

The effect of $[Ca]_o$ *on the time course of the potassium contracture*

Fast or twitch fibres. Contractures were induced by replacing the equilibrating solution with a sodium-free, potassium-rich solution (solution *E*, having 190 mM-K and unchanged Ca concentration). With 1·8 mM-Ca this contracture solution produces a rapid increase in tension, a plateau of several seconds, and a quick relaxation (Hodgkin & Horowicz, 1960*a*; for relevant work, concerning the effect of Ca on the time course of contractures in whole muscles, see Frank, 1960, and Pauschinger & Brecht, 1961). Figure 1 shows the effect of varying $[Ca]_o$. The most remarkable result of lowering $[Ca]_o$ from 4 to 0·1 mM was a shortening of the plateau. The rate of rise of tension and the rate of relaxation increased only slightly, and the height of the contractures dropped only after a reduction of $[Ca]_o$ to about 0·2 mM. In 'zero' Ca fluid no tension was produced. However, when changing from Ringer's solution with 1·8 mM-Ca to one without Ca, a period of 5–30 min was required before the fibre lost its capacity for developing any tension in response to an increase of $[K]_o$ (see p. 689).

In several experiments a comparison was made between the effects of 190 mM-K and the same concentration of Rb. During these tests $[Cl]_o$ was replaced by sulphate to ensure a strong depolarization with both kinds of ion, and 8 mM-$CaSO_4$ was added to obtain at least 1 mM of ionized Ca (Hodgkin & Horowicz, 1959). The fibres were left for 15 min in the equilibration solution containing (in mM): Na_2SO_4 40, $CaSO_4$ 8, K_2HPO_4 1·04,

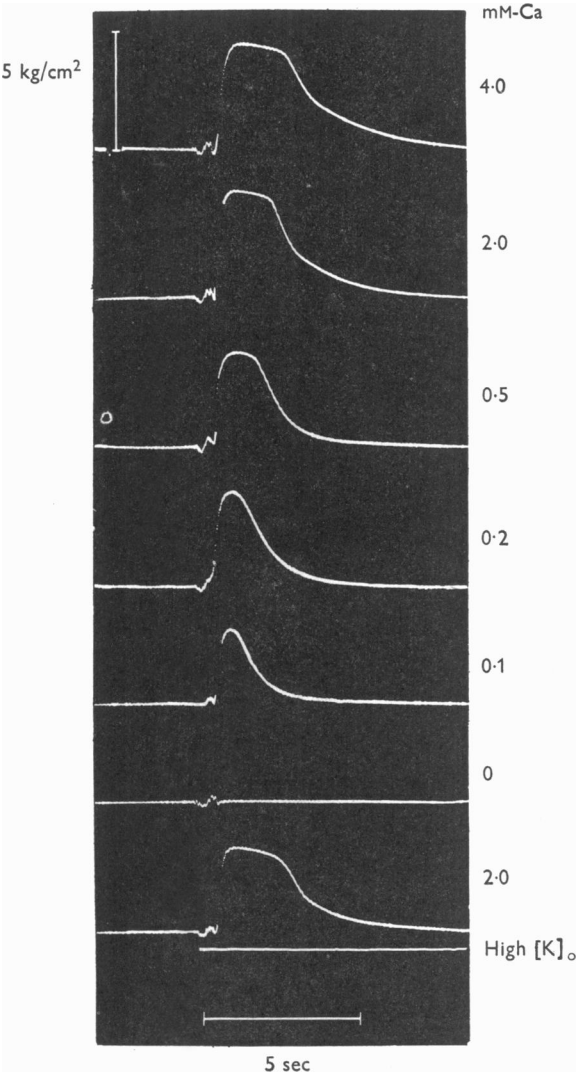

Fig. 1. Time course of the potassium contracture at varying values of $[Ca]_o$. The order of records was from top to bottom. The fibre was left for 10–15 min in Ringer's solution (solution *B*) for equilibration with the Ca concentration used during the following contracture. About 2 min before the contracture started, solution *B* was replaced by solution *C*, containing choline instead of Na to prevent twitches. The white line below the last contracture indicates the time during which the fibre was in high $[K]_o$. It was left in this solution (solution *E* with 190 mM-K) for about 10 sec. Iliofibularis, diameter 63 μ; the fibre was stretched to 1·25 times slack length.

KH_2PO_4 0·42, and sucrose 113, before they were immersed in the contracture solution with 95 mM-K_2SO_4 or Rb_2SO_4 instead of Na_2SO_4. The contractures elicited with either K or Rb were practically of the same strength and the same time course.

Slow fibres. A few experiments were carried out on single slow fibres from the tonus bundle of the iliofibularis (Sommerkamp, 1928; Kuffler & Vaughan Williams, 1953). These fibres were selected in the following way:

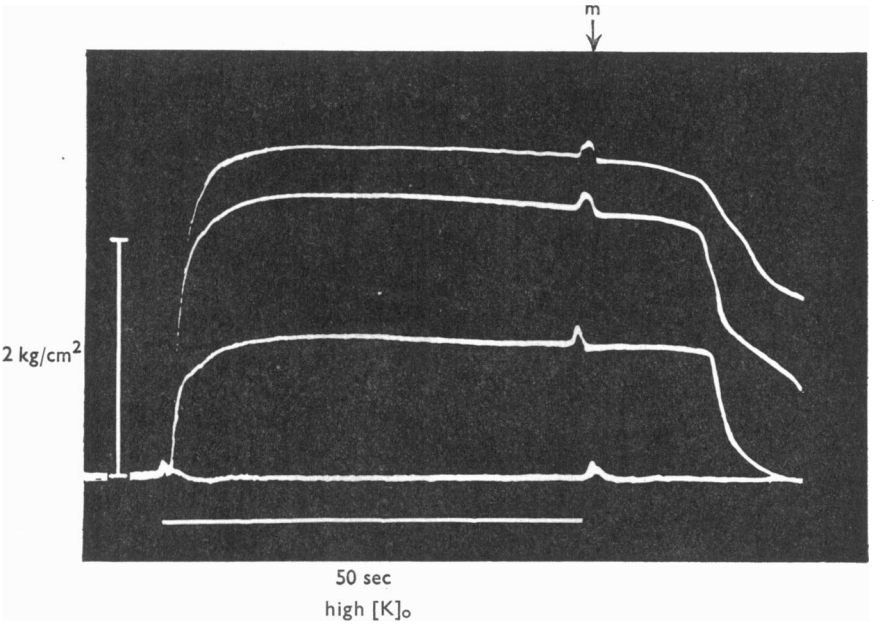

Fig. 2. Superimposed contractures of a single isolated slow fibre in solutions with varying calcium concentrations. The fibre was equilibrated for 15 min in Ringer's solution with the Ca concentration used in the following contracture and left for 50 sec (indicated by the white line at the bottom) in the contracture solution, which contained (mM): KCl 100, NaCl 117, $KHCO_3$ 2, $NaHCO_3$ 2, and varying amounts of $CaCl_2$. The order of records was from bottom to top. Ca concentrations: 0, 0·25, 2·0, and 4·0 mM. Iliofibularis; diameter 98 μ. The fibre was not stretched. m = mechanical artifacts marking the change from the contracture solution to Ringer's solution.

After the number of fibres was reduced during the dissection to about 10, a high-potassium solution was added, which caused a contracture in all fibres. Several seconds later, the twitch fibres relaxed and 'curled up' while other fibres remained in contracture. These were regarded as slow fibres and one of them was isolated (again in normal Ringer's solution) in the usual way. Contractures of such fibres in different $[Ca]_o$ are shown in Fig. 2. The contracture solution (solution *F*) contained the normal amount

of NaCl, since it is known that a mere reduction of $[Na]_o$ already induces contractures (Fleckenstein & Hertel, 1948; Schaechtelin, 1961).

The contractures of slow fibres differ from those of twitch fibres in several respects (see Schaechtelin, 1961, for corresponding results regarding the rectus abdominis):

(1) The rate of increase of tension is smaller.

(2) Practically no relaxation takes place in the high $[K]_o$ fluid.

(3) When the contracture solution is replaced by normal Ringer's solution, the tension remains high for about 10–20 sec before a quick and complete relaxation occurs.

(4) In fluids of reduced $[Ca]_o$ the fibres develop less tension, but are able to maintain this tension.

The tensions developed by 4 different fibres were: 1·4 (in 2 mM-Ca), 2·4 (2 mM-Ca), 2·7 and 2·8 (both 4 mM-Ca) kg/cm^2. The fibres were not stretched beyond their slack lengths, since it was found that stretching led to a reduction of tension. But the length which gives optimal contractures was not exactly estimated and it is possible that under more favourable conditions higher tensions may be developed by single slow fibres.

The effect of $[Ca]_o$ *on the relation between potassium concentration and peak tension*

Hodgkin & Horowicz (1960*a*) showed that development of tension in single twitch fibres is related to the log of $[K]_o$ by a steep S-shaped curve with tension starting at 20–30 mM-K. In this section experiments on twitch fibres are described which demonstrate the influence of $[Ca]_o$ on this relation. Figure 3 shows a typical result. Between contractures the fibres were left for 5 min in Ringer's solution (solution *B*) and for 10 min in choline Ringer's solution (solution *C*), both having the same Ca concentration as the contracture solution. Choline was used instead of Na to prevent action potentials and twitches. The contracture solutions consisted of a mixture of solutions *C* and *D*; i.e. choline was partly or fully replaced by K. Membrane potentials were not obtained simultaneously from the fibre under investigation, but from bundles of muscle fibres under the same condition.

After the tonus bundle had been removed from the iliofibularis muscle, membrane potentials were measured at 12, 22, 52, 102, and 190 mM-K, and the potentials indicated in Fig. 3 were extrapolated from a curve relating membrane potential to log $[K]_o$, which was constructed from values obtained at the above-mentioned potassium concentrations. For each point the mean value of 10 successful impalements in different fibres was taken. About 5 min was needed to achieve 10 determinations after increasing $[K]_o$. The potentials are lower than those published by Hodgkin & Horowicz (1960*a*), which were measured on single fibres simultaneously with tension. The difference might partly be explained by the continuous slow decrease of the membrane potential, due to the inward movement of KCl after the sudden elevation of the $[K]_o$ $[Cl]_o$ product (Hodgkin & Horowicz, 1959).

Even in low $[K]_o$ there was only a small difference in the membrane potential when the fibres were in 1·8 or 5·0 mM-Ca solutions (about e times increase of the calcium concentration, where e = base of Naperian logarithm). In 22 mM-K choline Ringer's solution the potentials were (mV): 5·0 mM-Ca $-50{\cdot}6 \pm 2{\cdot}6$ S.D.; 1·8 mM-Ca $-49{\cdot}8 \pm 1{\cdot}9$ S.D. (10 determinations each). These differences were neglected in Fig. 3, in which the membrane potential in 1·8 mM-Ca is shown on the abscissa.

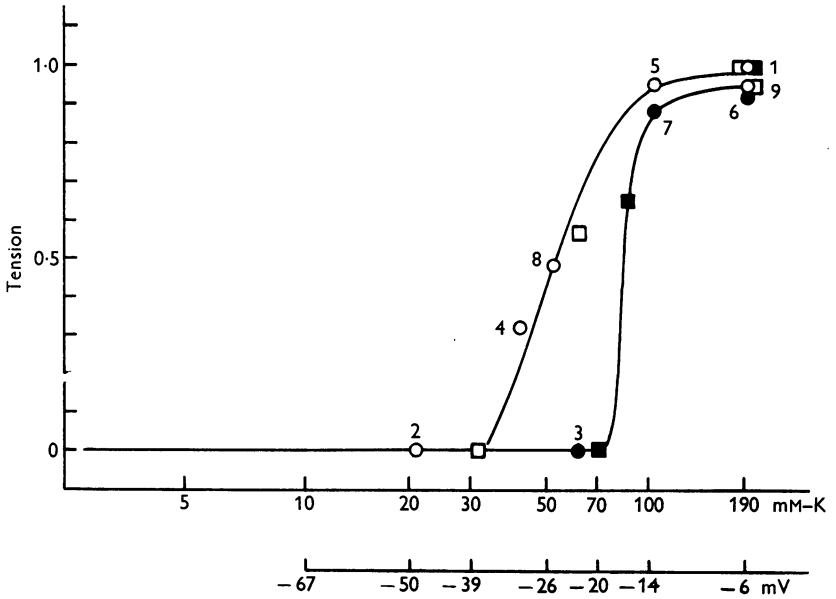

Fig. 3. Relation between peak tension and potassium concentration or membrane potential with two different Ca concentrations. Open symbols 1·8 and filled symbols 5·0 mM-Ca. 1·0 on the ordinate is equivalent to the height of the first contracture in 1·8 mM-Ca and 190 mM-K. The scale for the potassium concentration is logarithmic and for potential is approximately linear between −67 and −14 mV. The potential in 190 mM-K is still negative, probably owing to the relatively high Cl permeability of the muscle membrane. The membrane potential was estimated on small bundles of muscle fibres from the iliofibularis muscle. Choline Ringer's fluid with K replacing choline. ○, ●, fibre 34, diameter 65 μ, maximal tension 5·1 kg/cm². The numbers show the order in which the measurements were made. □, ■, fibre 36, diameter 70 μ, maximal tension 2·0 kg/cm². Fibres from the iliofibularis muscle.

In normal Ringer's solution containing 1·8 mM-Ca, contractures started at about 35 mM-K, reached half their maximum at 55 mM-K, and full maximum at 100 mM-K. An increase of $[Ca]_o$ raised the threshold of the potassium contracture as is seen by a shift of the log $[K]_o$–tension curve to the right. Also the slope of this curve is increased at the inflexion point, so the two curves differ by more than just a translational shift. With

5·0 mM-Ca contractures did not start below about 75 mM-K; they reached half maximum at about 80 mM-K and maximum with 100 mM-K. The shift of the threshold potential was from about -35 to -18 mV for this e times increase of $[Ca]_o$. In low Ca solutions (0·5 and 0·6 mM-Ca) weak contractures were already observed in the presence of 10–20 mM-K, but in 100 mM-K the contractures attained only 70–90 % of the maximal tension in normal $[Ca]_o$. With still higher potassium concentrations tension was sometimes even less (not included in Fig. 3).

The shift in threshold was first described by Fleckenstein & Hertel (1948) in whole gastrocnemius and rectus muscles. The threshold in normal $[Ca]_o$ of 20 mM-K rose to 50 mM-K after having increased $[Ca]_o$ from 1·8 to 53 mM. Orkand (1962) obtained corresponding results in single invertebrate muscle fibres (*Orconectes* and *Camburus*), using an electric current for depolarization.

The resting potential in Ca-free solutions

Figure 4 shows the effect of Ca-free Ringer's solution on the resting potential of isolated twitch fibres. The change of solutions took place in the usual rapid manner and a continuous slow flow of the Ca-free solution was maintained to avoid any possible accumulation of Ca ions leaving the fibre. A micro-electrode remained inside the fibre during this change of fluid. In 5 experiments the membrane potential immediately fell by about 10 mV when calcium was removed. This was followed by a slower decline in potential at a rate of about 1 mV/min. After 10–20 min in these Ca-free fluids the contractility of the fibres was tested by the application of a fluid containing 190 mM-K (solution *E* without Ca). Even with membrane potentials as low as -65 mV it was possible to induce a short contracture. After the contracture the fibres were left in normal Ringer's solution, in which recovery of the resting potential was slow and incomplete. In two experiments EDTA (1 mM) was added to the Ca-free solution. Its application induced spontaneous contractions and a fall in the resting potential, which was more rapid than that in the absence of EDTA. This fall in membrane potential was irreversible.

When Ca was replaced by 2 or 4 mM-Mg the membrane potential remained unchanged throughout the 20 and 30 min of investigation. A normal contracture could be elicited at the end of this time, but in both experiments that were made with Mg subsequent recovery of the resting potential was incomplete.

The results are in agreement with Ca and Mg experiments on superficial fibres in whole muscles by Edman & Grieve (1961) and Jenden & Reger (1962). The absence of the first sharp drop in potential, and the slower decline in potential following the removal of Ca in their experiments, can

probably be explained by the retarded decline of the Ca concentration in the intercellular space of the muscle.

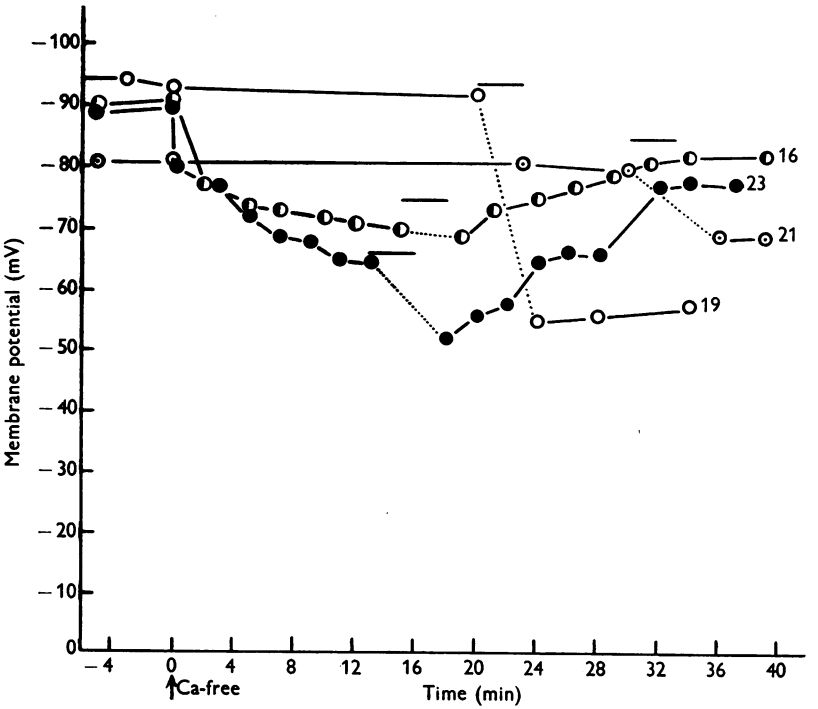

Fig. 4. Membrane potential of isolated fibres in Ca-free solutions. All fibres were in Ringer's solution until zero time, except fibre 21, which was left in Ringer's solution with 0·2 mM-Ca and 4·0 mM-Mg during the dissection. At zero time fibres 16 and 23 were immersed in Ca-free Ringer's solution, and fibres 19 and 21 in Ringer's solution in which Ca was replaced by 2 and 4 mM-Mg, respectively. The thick line above each curve indicates the time during which the fibre was in a Ca-free contracture solution with 190 mM-K. Throughout this time the micro-electrode was not inside the fibre, and the next point indicates the first measurement after a new impalement. The contracture solution was followed by Ringer's solution with 1·8 mM-Ca, only fibre 21 was immersed in a Ca-free Ringer's solution with 4 mM-Mg. ●, Fibre 23, Ca-free; ◐, fibre 16, Ca-free; ⊙, fibre 21, Ca-free, 4 mM-Mg; ○, fibre 19, Ca-free, 2 mM-Mg.

The effect of $[Ca]_o$ *on the steady relation between membrane potential and the state of the contractile system*

When a single twitch fibre is immersed in a solution containing 190 mM-K it develops tension for several seconds and then relaxes completely. On returning to Ringer's solution its capacity to contract is restored within tens of seconds. A full recovery can already be obtained in

Ringer's solution which still contains about 10 mM-K, but no restoration takes place in fibres which are immersed in a fluid with 50 or more mM potassium. Hodgkin & Horowicz (1960*a*) found an S-shaped relation between the logarithm of the external potassium concentration in which the fibres recovered after a contracture in 190 mM-K, and the state of the contractile system, tested by a second increase in $[K]_o$. In this section the effect of $[Ca]_o$ on this relation, i.e. on the amount of restoration, is described.

The procedure used by Hodgkin & Horowicz (1960*a*) was adopted: a contracture was induced by solution *E* (190 mM-K). After 10 sec this solution was replaced by choline Ringer's solution in which a certain amount of choline was substituted by potassium (mixture of solutions *C* and *D*). One minute later this test solution was again for 10 sec replaced by solution *E*, followed by a flow of normal Ringer's solution. It is assumed that after the first contracture the repriming or restoration of the contractile system reaches an equilibrium level within less than 1 min, but whether or not calcium influenced the speed of repriming was not tested. Between double contractures the fibres were left for 5 min in normal Ringer's solution and 10 min in choline Ringer's solution with the concentration of Ca used during the following contracture cycle. Figure 5 illustrates the result. On the abscissa is plotted the concentration of potassium (log. scale) in the test solution used between the two contractures and on the ordinate the height of the second contracture expressed as a percentage of the height of the first one, i.e. the amount of repriming. The figure gives the result from four fibres. The membrane potentials were taken from bundles of muscle fibres, as described on p. 683. Usually, the single fibres deteriorated after two to four double contractures. This might partly have been due to the increase in $[Cl]_i$, brought about while the fibre was in solutions with a high $[K]_o$ $[Cl]_o$ product. Double contractures were only evaluated when the first of them was nearly as large as that of the first contracture of the whole experiment. In normal $[Ca]_o$ the repriming reached 50 % at a K concentration of about 34 mM, which corresponded to a membrane potential of -37 mV. An e times increase in $[Ca]_o$ led to a shift of the curve towards higher $[K]_o$ with the half-maximum at 53 mM-K (-25 mV).

Mg ions

It was shown in a previous section that Mg ions can replace Ca in keeping the resting membrane potential at a normal value. In this section two additional sets of experiments are described which demonstrate that Mg has also similar effects to those of Ca on the contractile system.

It could be demonstrated in several experiments with single fibres that Mg causes a similar increase in tension threshold to that caused by Ca,

although no attempt was made to compare their effects quantitatively. A contracture solution with 1·8 mM-Ca and 60 mM-K, for example, produced a peak tension of about 100 mg in a fibre with a diameter of 90 μ. Addition of 4·2 mM-Mg to the equilibration and contracture solutions resulted in a failure of tension to be initiated by 60 mM-K, but after washing out the Mg ions a peak tension of 100 mg could again be obtained with the same potassium concentration.

Addition of Mg ions leads to an increased duration of the plateau of tension produced by 150 mM-K. For example, 8·2 mM-Mg, added to the equilibration and contracture solutions (with 1·8 mM-Ca), caused in one

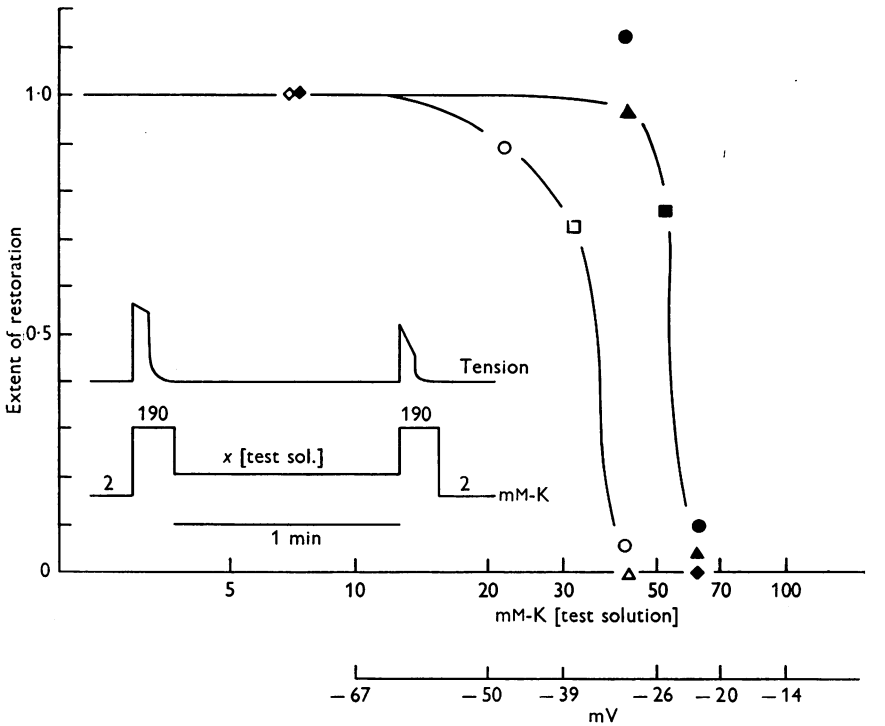

Fig. 5. Relation between potassium concentration and the state of the contractile system. The abscissa gives the potassium concentration of the test solution (which flowed through the experimental cell between the two contractures as indicated in the inset) on a logarithmic scale. The second scale gives the corresponding values of membrane potential. On the ordinate is plotted the height of the second contracture expressed as a percentage of the height of the first one, i.e. the extent of restoration. Open symbols 1·8 and filled symbols 5·0 mM-Ca. Fibres from the iliofibularis muscle. ○, ●, Fibre 30, diameter 111 μ, max. tension 3·0 kg/cm²; □, ■, fibre 31, diameter 75 μ, max. tension 5·2 kg/cm²; △, ▲, fibre 32, diameter 80 μ, max. tension 3·7 kg/cm²; ◇, ◆, fibre 33, diameter 90 μ, max. tension 3·8 kg/cm².

experiment a plateau of about 3 sec, whereas the plateaus in 1·8 mM-Ca alone and in 10 mM-Ca lasted 1·5 and 5 sec, respectively. All three contractures were obtained with the same fibre and subsequently repeated with nearly the same result. This experiment suggests that Mg ions act in a similar way to Ca ions, but are less effective, in agreement with the effects of these ions on the electrical properties of the squid axon membrane (Frankenhaeuser & Hodgkin, 1957).

The rate of action of calcium

This section deals with the rate of action of Ca and provides thus an idea about the region of the muscle fibre in which Ca causes the effects described previously.

A surprisingly long equilibration time of more than 20 min in Ca-free solutions was needed in several experiments, before development of tension was no longer observed. However, since only rather small amounts of Ca are required to preserve contractility, it is to be expected that Ca ions escaping from the fibre, and traces of this ion in the surrounding solution, may considerably influence the time during which contraction is not entirely abolished. This may explain the accelerating effect of EDTA (although the possibility that EDTA has additional effects in the membrane has to be considered, too), and, since in the present experiments no special precautions were taken to obtain really Ca-free fluids, it may have been the more drastically reduced extracellular Ca which caused the rather quick disappearance of contractility found by Frank (1960) in whole muscles of the frog's toe.

In this connexion it is of interest that elimination of Ca from normal Ringer's solution rapidly abolishes contractions of the frog's heart, whereas action potentials can be further obtained for rather a long time (Mines, 1913). The situation is, however, quite different when Ca *and* Na are omitted (NaCl replaced by sucrose). Under these circumstances contractions are possible for hours (van der Kloot & Rubin, 1962), presumably owing to the increased sensitivity towards Ca (Daly & Clark, 1921), so that small amounts of Ca produce maximal contractures.

In order to clarify this further two other sets of experiments were performed which avoid this and other difficulties of working with Ca-free solutions, and which provide more reliable information about the rate of action of calcium.

Since contractures do not allow a continuous testing of contractility, propagated action potentials followed by twitches were elicited by short cathodal pulses with a frequency of 1–2/sec, and the amplitude of the twitches was recorded in the usual way. In 'good' fibres a change of $[Ca]_o$ from 1·8 to 8 mM caused only a small reduction of twitch amplitude. With higher concentrations (10–15 mM) the height was reduced to about $\frac{1}{2}$ with

a half-time of 5–10 sec, and regained its normal value in 1·8 mM-Ca at the same rate. An increase of $[Ca]_o$ from 1·8 to 15 mM does not very much change the time course of the action potential (Ishiko & Sato, 1957), but it increases the threshold of excitation. Therefore, the possibility that the reduction of contraction is partly due to a block of conduction in certain regions of the fibre could not be completely excluded. Considering these limitations, the half-time of several seconds can only be regarded as a rough estimate of the rate of action of Ca.

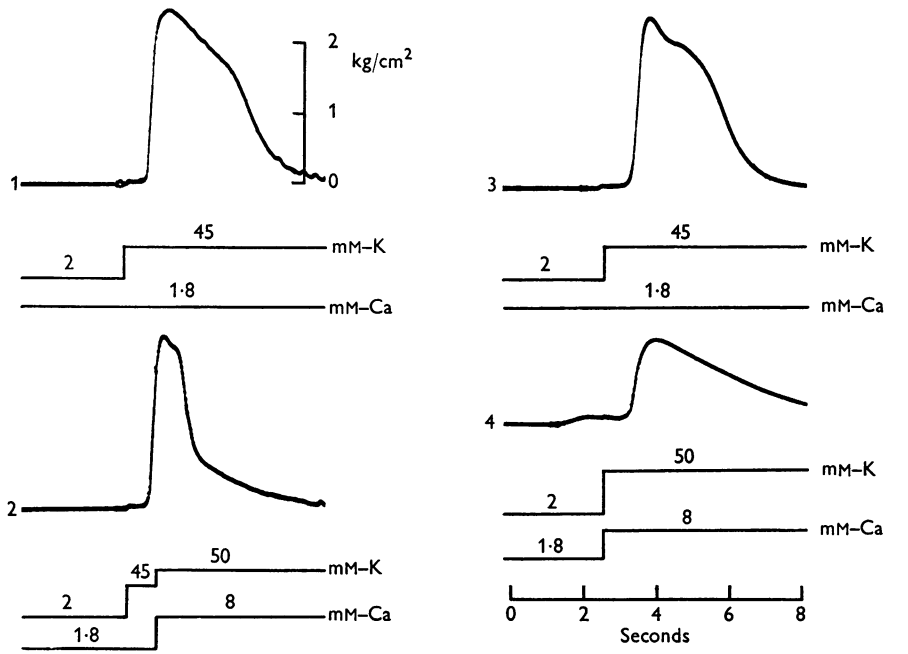

Fig. 6. The abolition of contracture by increasing $[Ca]_o$. Fibre 44, semitendinosus, diameter 108 μ. 2 min before every contracture Ringer's solution (solution *B*) was replaced by a Na-free choline Ringer's solution (*C*) to prevent twitches. The small deviation of the line in trace 4 before the solution exchange took place is regarded as a mechanical artifact.

Figure 6 describes a further experiment examining the rate of action of Ca. The first and the third contractures were elicited by increasing $[K]_o$ from 2 to 45 mM without changing $[Ca]_o$. The second contracture started with the same contracture solution, but when tension reached its maximum the solution was again changed so that $[K]_o$ increased to 50 mM and $[Ca]_o$ to 8 mM. This caused a sudden drop in tension which could not be accounted for by an increase in membrane potential, since the expected increase of potential after the elevation of $[Ca]_o$ was more than compensated for by an increase in $[K]_o$. The fourth trace shows that when $[Ca]_o$ and $[K]_o$ were

increased simultaneously to 8 and 50 mM respectively, the contracture reached only half the value of the preceding contractures.

The sudden relaxation after increasing $[Ca]_o$ can probably be explained by the increase in the mechanical threshold, which—according to Fig. 3—shifts from 30 to more than 70 mM-K when $[Ca]_o$ is increased from 1·8 to only 5 mM. The experiment suggests that the threshold increases immediately after elevation of $[Ca]_o$ and reaches its new steady state within several seconds.

DISCUSSION

The effect of Ca on the sodium-carrying system and on the system which controls contraction

The system that controls sodium conductance in excitable membranes and the effect of Ca on it has been extensively investigated during recent years (squid giant axon, Hodgkin & Huxley, 1952*a*, *b*; Frankenhaeuser & Hodgkin, 1957: heart, Weidmann, 1955: muscle fibres, Ishiko & Sato, 1957: myelinated nerve fibre, Frankenhaeuser, 1957). Several properties of this system are similar to those of the membrane control system for the contractile mechanism described by Hodgkin & Horowicz (1960*a*) and in this paper. It seems, therefore, reasonable to adopt for the latter the same terminology Hodgkin & Huxley (1952*a*, *b*) used to give an empirical description of how the Na conductance depends on time and membrane potential. The following survey of the similarities of action of Ca on both systems may be summarized by saying that qualitatively the changes in Ca concentration and changes in membrane potential have similar effects not only on the sodium-carrying system—as described by Frankenhaeuser & Hodgkin (1957)—but also on the system that controls contractility. To explain this parallel effect of Ca and potential, Professor Huxley (see Frankenhaeuser & Hodgkin, 1957) made the suggestion that Ca (and Mg) ions may be adsorbed at the outer edge of the membrane and thus increase the electric field inside it. This adsorption hypothesis might also explain why certain anions act in the opposite way to Ca. Thus, it was found that the replacement of extracellular Cl by NO_3 or SCN reduced the contraction threshold of twitch fibres, and that the potentiating effects of anions on the twitch are in the order $Cl < Br < NO_3 < I < SCN$, which corresponds to the increasing adsorbability of these anions (Kahn & Sandow, 1950; Hodgkin & Horowicz, 1960*b*).

Frankenhaeuser & Hodgkin (1957) showed in voltage-clamp experiments that when $[Ca]_o$ was increased the curve relating peak Na conductance to membrane potential was shifted along the voltage axis in a direction such that a stronger depolarization was required to increase the Na conductance to a given size. This result provides a reasonable explanation

for the increase in threshold for the action potential which was observed in various cells; e.g. Weidmann (1955) found in *Purkinje* fibres a shift in threshold potential from -73 to -65 mV after increasing $[Ca]_o$ by a factor of 4. This shift is comparable to the shift in tension threshold in twitch fibres. For tension, however, no all-or-nothing reaction exists, as there is no process resembling the regenerative increase of the sodium conductance during an action potential.

In the steady state increasing $[Ca]_o$ at constant membrane potential decreased the percentage of the sodium-carrying system which is in an inactive or refractory condition. Weidmann (1955) observed a shift of the S-shaped curve, relating peak sodium current to membrane potential, of 5–6 mV towards depolarization when $[Ca]_o$ was increased 4 times. This corresponds to a similar shift of the steady-state peak tension as described in this paper. In the squid axon Frankenhaeuser & Hodgkin (1957) noticed that increasing $[Ca]_o$ reduces the rate of inactivation under a cathode and increases the rate at which inactivation is removed under an anode. Both effects give a reasonable explanation for the described shift of the inactivation curve. No exact statements are as yet possible about the rate constants of inactivation and reactivation of the contraction-activating system and their dependence on $[Ca]_o$. However, the same shift of the inactivation curve and the prolongation of the plateau of contracture after increasing $[Ca]_o$ exhibit a similar mechanism.

This comparison leads to the tentative suggestion that Ca influences the shift of an activator of contraction from the outside to the inside of the membrane in essentially the same way that it influences sodium transport. The fact that the system is only slightly affected when the external Na is replaced by choline (Hodgkin & Horowicz, 1960*a*) and that the rate of contraction inactivation is about 1000 times slower than the rate of inactivation of the Na transport make it rather unlikely that Na is the activator of contraction. Although there is no direct evidence for or against such a view, it might be possible that Ca itself or a negatively charged Ca complex starts contraction by moving to the inside of the membrane, presumably in some part of the endoplasmic reticulum (Porter & Pallade, 1957; Bianchi & Shanes, 1959; Hodgkin & Horowicz, 1960*a*, *b*). The latter assumption is supported by the experiments which show that after changing $[Ca]_o$ several seconds are necessary to reach a new twitch height or a new tension threshold. The rate of action is thus rather quick, but slower than, for example, that of Na on the action potential. Foreign anions act in a similar slow way on twitch amplitude (Hodgkin & Horowicz, 1960*b*). This can be explained by assuming that the ions do not act directly at the surface membrane, but have to diffuse into the tubules of the endoplasmic reticulum (Hodgkin & Horowicz, 1960*a*, *b*).

The failure of contraction in Ca-free solutions

The experiments described in this paper confirm the conclusion of Jenden & Reger (1962) that the disappearance of contraction upon removal of extracellular Ca is due to a combined effect of a progressive decline in resting potential and a shift of the S-shaped inactivation curve, relating the steady state of the contractile system to membrane potential, towards hyperpolarization. Theoretically, an inactivation of the contractile system at a normal resting potential is possible. However, such a situation was never observed in experiments in which the resting potential was measured with internal electrodes (Edman & Grieve, 1961; Jenden & Reger, 1962; this paper, p. 685). Fibres in zero Ca Ringer's solution with normal or slightly reduced resting potentials always developed tension when suddenly depolarized by increasing $[K]_o$.

Mg ions can replace Ca, at least for the relatively short times used in the present experiments, but it is uncertain whether they can completely restore a normal resting potential as well as a normal contracture after a depolarization due to a lack of Ca or an increase in $[K]_o$. On whole toe muscles Frank (1962) found that several multivalent cations, including Be, Mg, and Zn, in the absence of Ca can restore the ability to develop tension after an increase in $[K]_o$, but in every case only for a limited time. Since the membrane potential was not measured, it remains uncertain whether or not the final failure of contracture was due to depolarization.

Differences between heart and twitch muscle fibres

Although Ca ions are necessary for the normal contraction of heart and twitch muscle fibres, there are great differences between the membrane systems which activate contraction.

(1) Increasing $[K]_o$ or depolarization evokes a phasic contracture in twitch fibres (Hodgkin & Horowicz, 1960*a*) and a lasting contracture in the heart (Niedergerke, 1956). In high $[Ca]_o$ the duration of the plateau of the phasic contracture becomes longer, whereas in heart fibres an increase of tension throughout the whole contracture time takes place.

(2) Ca increases the threshold of tension in twitch fibres (Lüttgau, 1962) and reduces it in heart fibres (Niedergerke, 1956). In the latter a maximal contracture can be produced without lowering the resting potential, merely by drastically increasing $[Ca]_o$.

(3) Mg acts in a similar way to Ca in twitch fibres (Jenden & Reger, 1962), but not in the heart (Lüttgau & Niedergerke, 1958).

A reduction of $[Na]_o$ has little effect in twitch fibres (Hodgkin & Horowicz, 1960*a*), whereas in the heart it augments the effect of Ca. The

height of contraction is quantitatively related to the ratio $[Ca]_o:[Na]_o^2$ (Wilbrandt & Koller, 1948).

(5) Caffeine, added to Ringer's solution, can without depolarization produce contractures in twitch fibres (Axelsson & Thesleff, 1958), but not in the heart (Suzuki, 1962).

These results, together with Ca-flux measurements (Niedergerke, 1959; Winegrad & Shanes, 1962), indicate that after a normal excitation of the heart an inward movement of Ca may initiate contraction. Removal of extracellular Ca consequently breaks the link which couples electrical and mechanical events. In twitch muscle fibres a more efficient and elaborate excitation–contraction coupling system seems to exist, in which the most striking effect of extracellular Ca consists in 'stabilizing' membrane processes which lead to contraction.

Although it is impossible at the present stage to develop in detail a hypothesis of excitation–contraction coupling in twitch fibres, a few experiments, connected with this problem, should be mentioned:

(1) Huxley & Taylor (1958) and Huxley & Straub (1958) demonstrated in frog and lizard muscles with extracellular micropipettes that the region which contracts as a single unit is centred on a triad. They concluded that some component of the triad may be the structure along which membrane depolarization during an activation is conducted inwards.

(2) Heilbrunn & Wiercinski (1947) and Niedergerke (1955) have shown in frog fibres, and Caldwell & Walster (1961) in crab fibres that the injection of a small amount of Ca into muscle cells causes contracture, whereas Mg has no effect. The same specific action of Ca was observed by Podolsky & Hubert (1961), who induced contractures in isolated fibrils.

(3) Hasselbach and co-workers (Hasselbach, 1962) have shown that the isolated granules or vesicles of the relaxing factor, which are able to take up and store Ca, resemble in structure some elements of the endoplasmic reticulum and may conceivably be identical with triads.

From these experiments and those described in this paper it might tentatively be suggested that the proposed 'activator' in the reticulum may cause the release of Ca ions from Ca-storing parts of the triad into the fibre, and thus initiate contraction in the same way as artificially injected calcium.

Slow fibres seem to behave very similarly to heart fibres (see Schaechtelin, 1961), but since little information on single fibres is available they have been excluded from the discussion. It should, however, be mentioned that triads, which seem to play an important part in excitation–contraction coupling in twitch fibres, are missing (Peachey, 1961).

SUMMARY

1. The effects of external calcium ions on the isometric contracture of isolated twitch muscle fibres from the semitendinosus and iliofibularis muscles of the frog were recorded with a mechano-electrical transducer. In addition, membrane potentials were measured with glass microelectrodes.

2. A sudden increase of $[K]_o$ from 2 to 190 mM caused a quick rise of tension to a maximal level, which was maintained for several seconds (plateau) and followed by a rapid relaxation. Reducing $[Ca]_o$ from 4·0 to 0·2 mM brought about a shortening of the plateau without affecting peak tension.

3. Fibres which were left for 5–30 min in Ca-free Ringer's solution no longer developed tension on increasing $[K]_o$.

4. Raising $[Ca]_o$ caused a shift to higher potassium concentrations and an increase in steepness of the S-shaped curve which relates peak tension to log $[K]_o$ or membrane potential. An increase of $[Ca]_o$ from 1·8 to 5 mM changed the mechanical threshold from 35 to 75 mM-K (-35 to -18 mV).

5. In the steady state the inactivation of contracture tension is related to log $[K]_o$ or membrane potential by an S-shaped curve, whose half-value shifted from 34 to 53 mM-K (-37 to -25 mV) after increasing $[Ca]_o$ from 1·8 to 5 mM.

6. The actions of Mg ions on these mechanical characteristics are similar to those of Ca ions, though probably not as strong.

7. Application of Ca-free Ringer's solution caused a sudden drop in resting potential by about 10 mV and a further decline at a rate of about 1 mV/min.

8. A few contracture experiments were carried out with slow fibres from the tonus bundle of the iliofibularis. Elevation of $[K]_o$ led to a slow increase in tension, the maximum of which was maintained throughout the time (50 sec) of application of high $[K]_o$. The strength of tension depended on $[Ca]_o$: at zero $[Ca]_o$ no tension was developed, while with 4–6 mM-Ca tension attained a saturation value.

9. It is concluded that the actions of $[Ca]_o$ on the system which activates contraction in twitch fibres are quite unlike those in the heart but resemble the stabilizing effects of Ca or Mg on the electrical properties of excitable membranes.

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